DESCRIPTION

METHOD FOR DETECTING GENE AFFECTED BY ENDOCRINE DISRUPTOR

INSA1

Technical Field

The present invention relates to methods for detecting an endocrine disruptor which influences homeostasis of a living body, and a gene influenced by said substance.

Background Art

Subc1

Endocrine disruptors (often referred to as environmental hormones) collectively refer to chemical substances released in environment for which hormone-like activities or anti-hormone activities have been found. Altered reproductive potential (in particular, conversion of male into female), decreased reproductive potential, decreased hatchability, decreased survival rate of offspring, abnormal reproductive behavior and the like have been reported to be resulted from the influences of endocrine disruptors on the ecosystem of wild animals. Decreased number of sperms, endometriosis, infertility, ovarian cancer, uterine cancer, prostatic cancer and the like have been suspected to be resulted from the influences of endocrine disruptors on human health, although they have

~~not been demonstrated.~~

Substances (or groups of substances) that are considered to cause endocrine disruption are reported in the interim report (July, 1997) by "Exogenous Endocrine Disrupting Chemical Task Force" of Environment Agency. However, it is considered that the types of such substances would be further increased in the course of research and study in the future.

Known methods for determining endocrine disruptors are classified into two groups, i.e., in vitro methods and in vivo methods. Examples of the methods in the former group include a method in which a binding activity to estrogen receptor or androgen receptor is measured, and a method in which an activity of inhibiting a hormone synthesis enzyme system is measured. Examples of the methods in the latter group include a method in which production of various hormones and abnormal tissue formation in rats at different postnatal days are measured, a method in which abnormal metamorphosis in a frog is measured, and a method in which abnormal maturation in a fish is measured (Analytical Chemistry, 70(15):528A-532A (1998)).

However, it has not been clearly demonstrated to date whether or not the substances that are suspected to be endocrine disruptors with attention actually cause

endocrine disruption. Furthermore, if they cause endocrine disruption, the mechanism through which they influence as well as the amount and the length of the period of intake that might be risky have not been clearly demonstrated.

For example, the current binding test to a hormone receptor is necessary and important as a primary screening. However, the results obtained by this method do not guarantee the identity as an endocrine disruptor. Specifically, estradiol (a naturally occurring female hormone), diethylstilbestrol (a synthetic female hormone), isoflavone (a component contained in pulses which is harmless to humans) and bisphenol-A (a substance suspected to be an endocrine disruptor) bind to estrogen receptor, although the EC_{50} values for these substances are different each other. Thus, the degree of endocrine disrupting activity of each substance cannot be determined according to this assay method. Similarly, the activity cannot be determined according to any of the conventional methods including an assay system in which a yeast or a cultured cell is used, and a system in which uterine of a mouse is weighed.

In other words, the current methods in which a binding activity to a hormone receptor or an activity of inhibiting hormone synthesis enzyme system is measured in vitro meet a necessary condition as a method for measuring

an endocrine disruptor. However, they never meet a sufficient condition. Furthermore, methods in which influences on the growth or morphogenesis of a rat, a frog, a fish or the like are determined in vivo are less sensitive and complicated, and require a long period of time for operating a large number of samples.

In addition, although the conventional analysis methods as described above may be used to analyze the relationship between a potential endocrine disruptor and a receptor, they cannot be used to analyze the downstream signal transduction system.

As described above, it is necessary for solving problems of environmental hormones to identify endocrine disruptors and to determine the influences by the substances on the endocrine systems. Thus, methods for analyzing which signal transduction pathway is influenced by an endocrine disruptor and which substance causes endocrine disruption have been desired.

Objects of Invention

The main object of the present invention is to provide (1) a method for detecting a gene that is influenced by an endocrine disruptor; (2) a method for detecting a gene that is influenced by an endocrine disruptor which comprises measuring the expression of the

gene detected by said method; (3) a DNA array onto which a gene that is influenced by an endocrine disruptor or a DNA fragment derived from the gene is immobilized; and (4) a method for detecting a substance that potentially causes endocrine disruption.

Summary of Invention

As a result of intensive studies, the present inventors have constructed a method for detecting many types of genes that are influenced by endocrine disruptors rapidly, with high sensitivity and simultaneously. The present inventors have found a method for detecting endocrine disruptors using a DNA array onto which said genes or fragments thereof are immobilized. Furthermore, the present inventors have constructed a method for detecting a substance that potentially causes endocrine disruption. Thus, the present invention has been completed.

In summary, the present invention relates to:

[1] a method for detecting a gene that is influenced by an endocrine disruptor, characterized in which the method comprises:

preparing a nucleic acid sample containing mRNAs, or cDNAs therefor, derived from a cell, a tissue or an organism which has been exposed to a sample containing an endocrine disruptor;

hybridizing the nucleic acid sample with a DNA array onto which genes which are potentially influenced by the endocrine disruptor or DNA fragments derived from the genes which are potentially influenced by the endocrine disruptor are immobilized; and

selecting a gene that is influenced by the endocrine disruptor by comparing the results with results for a nucleic acid sample prepared using a control sample;

[2] the method according to [1] above, wherein a gene selected from the group consisting of:

(1) a gene for a nuclear receptor or a gene related to nuclear receptor transcriptional coupling;

(2) a gene related to kinase-type signal transduction;

(3) a gene related to gonad differentiation;

(4) a gene for or related to a receptor-type kinase;

(5) a gene for or related to an intermediate filament marker;

(6) a gene related to cell cycle or growth regulation;

(7) an oncogene, a gene related to an oncogene or a gene related to tumor suppression;

(8) a gene related to apoptosis;

(9) a gene related to damage response, repair or

recombination of DNA;

(10) a gene for or related to a receptor;

(11) a gene related to cell death or differentiation regulation;

(12) a gene related to adhesion, motility or invasion of cell;

(13) a gene related to angiogenesis promotion;

(14) a gene related to cellular invasion;

(15) a gene related to cell-cell interaction;

(16) a gene for or related to a Rho family, GTPase or a regulator therefor; and

(17) a gene for or related to a growth factor or a cytokine,

or a DNA fragment derived from the gene is used;

[3] a method for detecting an endocrine disruptor, characterized in which the method comprises measuring the expression of the gene detected by the method according to [1] or [2] above;

[4] the method according to [3], wherein the endocrine disruptor is selected from ones classified into:

(1) dioxins;

(2) organochlorine compounds;

(3) phenols;

(4) phthalate esters;

(5) aromatic hydrocarbons;

(6) pesticides;

(7) organotin compounds;

(8) estrogens; or

(9) mirex, toxaphene, aldicarb or kepone;

[5] a method for detecting a substance that potentially causes endocrine disruption, characterized in which the method comprises:

preparing a nucleic acid sample containing mRNAs, or cDNAs therefor, derived from a cell, a tissue or an organism which has been exposed to a sample that is suspected to contain a substance that potentially causes endocrine disruption;

hybridizing the nucleic acid sample with a DNA array onto which genes which are influenced by an endocrine disruptor or DNA fragments derived from the genes which are influenced by the endocrine disruptor are immobilized; and

detecting a substance that potentially causes endocrine disruption by comparing the results with results for a nucleic acid sample prepared using a control sample;

[6] the method according to [5] above, wherein the substance that potentially causes endocrine disruption is classified into:

(1) dioxins;

(2) organochlorine compounds;

(3) phenols;

(4) phthalate esters;

(5) aromatic hydrocarbons;

(6) pesticides;

(7) organotin compounds;

(8) estrogens; or

(9) mirex, toxaphene, aldicarb or kepone;

[7] a DNA array for detecting a gene that is influenced by an endocrine disruptor, onto which a gene that is influenced by an endocrine disruptor or a gene that is potentially influenced by an endocrine disruptor, or a DNA fragment derived from the gene is immobilized;

[8] the DNA array according to [7] above, onto which a gene selected from the group consisting of:

(1) a gene for a nuclear receptor or a gene related to nuclear receptor transcriptional coupling;

(2) a gene related to kinase-type signal transduction;

(3) a gene related to gonad differentiation;

(4) a gene for or related to a receptor-type kinase;

(5) a gene for or related to an intermediate filament marker;

(6) a gene related to cell cycle or growth regulation;

(7) an oncogene, a gene related to an oncogene or

a gene related to tumor suppression;

(8) a gene related to apoptosis;

(9) a gene related to damage response, repair or recombination of DNA;

(10) a gene for or related to a receptor;

(11) a gene related to cell death or differentiation regulation;

(12) a gene related to adhesion, motility or invasion of cell;

(13) a gene related to angiogenesis promotion;

(14) a gene related to cellular invasion;

(15) a gene related to cell-cell interaction;

(16) a gene for or related to a Rho family, GTPase or a regulator therefor; and

(17) a gene for or related to a growth factor or a cytokine,

or a DNA fragment derived from the gene is immobilized; and

[9] the DNA array according to [7] or [8] above, wherein the gene or the DNA fragment derived from the gene is immobilized onto a slide glass.

Detailed Description of the Invention

(1) The method for detecting a gene that is influenced by an endocrine disruptor of the present invention

As used herein, an endocrine disruptor (also referred to as an exogenous endocrine disruptor or an environmental hormone) means an exogenous substance which, when incorporated into a living body of an animal, influences a normal activity of a hormone naturally exerted in the living body. The endocrine disruptors include ones that maintain, promote or suppress a normal activity of a hormone. Substances that potentially influence a normal activity of a hormone are also included within the definition.

Currently, about 70 substances (or groups of substances) are suspected to have endocrine disrupting activities. These substances are classified in the materials for the abstract of 24th Meeting of the Japan Society for Environmental Chemistry (1998) as follows based on the methods for analyzing the corresponding substances: (1) Category 1: organochlorine compounds (e.g., general organochlorine compounds, polychlorinated biphenyl (PCB)); (2) Category 2: phenols (e.g., general phenols, bisphenol-A, 2,4-dichlorophenol, pentachlorophenol), (3) Category 3: phthalate esters (e.g., general phthalate esters); (4) Category 4: aromatic hydrocarbons (e.g., benzo(a)pyrene, di-2-ethylhexyl adipate (DEHA), benzophenone, 4-nitrotoluene, stylene dimer and trimer, 1,2-dibromo-3-chloropropane, styrene, n-butylbenzene); (5) Category 5:

pesticides (e.g., general pesticides, 2,4,5-T (trichlorophenoxyacetic acid), 2,4-D (dichlorophenoxyacetic acid), benomyl, amitrole, methomyl); (6) Category 6: organotin compounds; (7) Category 7: estrogens; dioxins which are not classified into the above-mentioned seven categories; and substances which are excluded from the categories, i.e., mirex, toxaphene, aldicarb and kepone (chlordecone). These categories and the substances are shown in Table 1.

Table 1

Category/Substance	
Category 1	Category 3
Polychlorinated biphenyl (PCB)	Di-2-ethylhexyl phthalate
Polybrominated biphenyl (PBB)	Butylbenzyl phtalate
Hexachlorobenzene (HCB)	Di-n-butyl phthalate
Hexachlorocyclohexane	Dicyclohexyl phthalate
Chlordane	Diethyl phthalate
Oxychlordane	Dipentyl phthalate
trans-Nonachlor	Dihexyl phthalate
DDT	Dipropyl phthalate
DDE, DDD	
Kelthane	Category 4
Aldrin	1,2-Dibromo-3-chloropropane
Endrin	Benzo(a)pyrene
Dieldrin	Di-2-ethylhexyl adipate
Endosulfan (benzoepin)	Benzophenone
Heptachlor	4-Nitrotoluene
Heptachlor epoxide	Stylene dimer and trimer
Methoxychlor	Styrene
Octachlorostyrene	n-Butylbenzene
Category 2	Category 5
Pentachlorophenol (PCP)	2,4,5-Trichlorophenoxyacetic acid
Alkylphenol (C5-C9)	2,4-Dichlorophenoxyacetic acid
Bisphenol-A	Amitrole
2,4-dichlorophenol	Atrazine

Category 5 (continued)	Category 6
Alachlor	Tributyltin
Simazine	Triphenyltin
Ethylparathion	
	Category 7
Carbaryl	
	Estradiol
Malathion	
Methomyl	
Nitrophene	Not classified into the categories
Trifluralin	
Benomyl	Dioxin
Manzeb (mancozeb)	
	Environmental hormones excluded from the categories
Maneb	
Metiram	
Metribuzin	Mirex
Cypermethrin	Toxaphene
Esfenvalerate	Aldicarb
Fenvalerate	Kepone (chlordecone)
Permethrin	
Vinclozolin	
Zineb	
Ziram	

The endocrine disruptors are not limited to those listed above. For example, diethylstilbestrol (DES) which is known to cause vaginal cancer in humans, and bisphenol-A for which an estrogen (female) activity and toxicity have been observed are considered to have endocrine disrupting activities.

These substances are known to have the following primary activities: 1) direct activities on hormone receptors (e.g., synthetic hormone formulations, DDT, phthalate esters, etc.); 2) activities through other receptors (e.g., dioxins, etc.); 3) activities of inhibiting metabolism (e.g., steroid metabolic inhibitors, inhibitors of aromatase or 5 α-reductase, etc.); and 4) activities through other systems (e.g., substances that influence nervous system or immune system). Thus, their modes of actions are diverse [Kagaku (Chemistry), 53(7):12-15 (1998)].

As used herein, a gene that is influenced by an endocrine disruptor is defined as a gene of which the expression is promoted or suppressed by the above-listed endocrine disruptor as compared with a control. The number of the gene(s) may be one, or two or more. Thus, a gene for or related to an agent of which the expression is directly and/or indirectly influenced by an endocrine disruptor may be selected as the gene to be immobilized onto the DNA array of the present invention. Genes of which the expression is promoted and genes of which the expression is suppressed can be preferably used. Examples of preferable candidates for such genes (i.e., genes that are potentially influenced by endocrine disruptors) include, but are not limited to, those shown in Table 2 which are

classified as follows: (1) a gene for a nuclear receptor or a gene related to nuclear receptor transcriptional coupling (nuclear receptor or nuclear receptor transcriptional coupling); (2) a gene related to kinase-type signal transduction (kinase-type signal transduction); (3) a gene related to gonad differentiation (gonad differentiation); (4) a gene for or related to a receptor-type kinase (receptor-type kinase); (5) a gene for or related to an intermediate filament marker (intermediate filament markers); (6) a gene related to cell cycle or growth regulation (cell cycle & growth regulators); (7) an oncogene, a gene related to an oncogene or a gene related to tumor suppression (oncogenes & tumor suppressors); (8) a gene related to apoptosis (apoptosis); (9) a gene related to damage response, repair or recombination of DNA (DNA damage response, repair & recombination); (10) a gene for or related to a receptor (receptors); (11) a gene related to cell death or differentiation regulation (cell fate & development regulators); (12) a gene related to adhesion, motility or invasion of cell (cell adhesion, motility & invasion); (13) a gene related to angiogenesis promotion (angiogenesis regulators); (14) a gene related to cellular invasion (invasion regulators); (15) a gene related to cell-cell interaction (cell-cell interactions); (16) a gene for or related to a Rho family, GTPase or a regulator

therefor (Rho family small GTPases & their regulator); and (17) a gene for or related to a growth factor or a cytokine (growth factors & cytokines). Furthermore, other genes for or related to agents of which the expression is potentially influenced by endocrine disruptors in a direct and/or indirect manner are also included in the genes that are influenced by endocrine disruptors of the present invention.

Table 2

Gene Name	GenBank Accession #(s)	Classification
type I cytoskeletal 10 keratin; cytokeratin 10 (K10)	X14487	intermediate filament markers
cell division control protein 2 homolog (EC 2.7.1.-);cyclin-dependent kinase 1 (CDK1)	X05360	cell cycle & growth regulators
cell division protein kinase 4 (EC 2.7.1.-) (PSK-J3)	M14505	cell cycle & growth regulators
type I cytoskeletal 13 keratin; cytokeratin 13 (K13; CK 13)	X14640	intermediate filament markers
type I cytoskeletal 14 keratin; cytokeratin 14 (K14; CK 14)	J00124	intermediate filament markers
type I cytoskeletal 18 keratin; cytokeratin 18 (K18)	M26326	intermediate filament markers
type I cytoskeletal 19 keratin; cytokeratin 19 (K19; CK 19)	Y00503	intermediate filament markers
cyclin-dependent kinase 5 activator precursor (CDK5 activator)	X80343	cell cycle & growth regulators
cell division cycle protein 25A tyrosine phosphatase (cdc25A); M-phase inducer phosphatase 1 (EC 3.1.3.48)	M81933	cell cycle & growth regulators
CDC25B; M-phase inducer phosphatase 2 (EC 3.1.3.48)	S78187	cell cycle & growth regulators
cdc25C; M-phase inducer phosphatase 3 (EC 3.1.3.48)	M34065	cell cycle & growth regulators
CLK-2	L29218	cell cycle & growth regulators
CLK-3	L29220	cell cycle & growth regulators
serine/threonine-protein kinase KKIALRE	X66358	cell cycle & growth regulators
type II cytoskeletal 11 keratin; cytokeratin 1 (K1; CK 1); 67-kDa cytokeratin; hair alpha protein	M98776	intermediate filament markers
CDC2-related protein kinase CHED	M80629	cell cycle & growth regulators
cdc2-related protein kinase PISSLRE	L33264	cell cycle & growth regulators
cyclin A	X51688	cell cycle & growth regulators
type II cytoskeletal 4 keratin; cytokeratin 4 (K4; CK4)	X07695	intermediate filament markers
cyclin C G1/S-specific	M74091	cell cycle & growth regulators

type II cytoskeletal 5 keratin; cytokeratin 5 (K5; CK 5); 58-kDa cytokeratin	M21389	intermediate filament markers
cyclin D2	D13639	cell cycle & growth regulators
cyclin E	M73812	cell cycle & growth regulators
cyclin G1	X77794	cell cycle & growth regulators
cyclin G2	U47414	cell cycle & growth regulators
type II cytoskeletal 6 keratin: cytokeratin 6B (CK 6B); K6B keratin	L42610	intermediate filament markers
AMP deaminase isoform L (AMPD2) mRNA	M91029	cell cycle & growth regulators
type II cytoskeletal 7 keratin; cytokeratin 7 (K7; CK 7)	M13955	intermediate filament markers
CDK6 inhibitor 2c (p18) mRNA, complete cds	U17074	cell cycle & growth regulators
type II cytoskeletal 8 keratin; cytokeratin 8 (K8; CK 8)	X74929	intermediate filament markers
p35 cyclin-like CAK1-associated protein	X87843	cell cycle & growth regulators
wee1Hu CDK tyrosine 15-kinase; wee-1-like protein kinase	U10564	cell cycle & growth regulators
serine/threonine-protein kinase PLK (EC 2.7.1.-) (PLK-1) (STPK13)	U01038	cell cycle & growth regulators
phospholipase D1 (PLD 1); choline phosphatase 1	U38545	cell cycle & growth regulators
CDC10 protein homolog	S72008	cell cycle & growth regulators
CDC27HS Protein	S78234	cell cycle & growth regulators
CDC16HS	U18291	cell cycle & growth regulators
CDC37 homolog	U43077	cell cycle & growth regulators
CDC6-related protein	U77949	cell cycle & growth regulators
extracellular signal-regulated kinase 1 (ERK1)	X60188	cell cycle & growth regulators
extracellular signal-regulated kinase 2 (ERK2)	Z11695	cell cycle & growth regulators
extracellular signal-regulated kinase 3 (ERK3)	X80692	cell cycle & growth regulators
extracellular signal-regulated kinase 4 (ERK4)	X59727	cell cycle & growth regulators
extracellular signal-regulated kinase 5 (ERK5)	U29726	cell cycle & growth regulators
mitogen-activated protein kinase p38 (MAP KINASE p38)	L35263	cell cycle & growth regulators
vimentin	Z19554	intermediate filament markers
profilin	J03191	intermediate filament markers

c-jun N-terminal kinase 3 (JNK3)	U34819	cell cycle & growth regulators
dual specificity mitogen-activated protein kinase kinase 5	U25265	cell cycle & growth regulators
dual-specificity mitogen-activated protein kinase kinase 1	L11284	cell cycle & growth regulators
dual-specificity mitogen-activated protein kinase kinase 6	U39065	cell cycle & growth regulators
PCNA; cyclin	M15796	cell cycle & growth regulators
retinoblastoma-binding protein (RBP)	S66427	cell cycle & growth regulators
RBQ1 retinoplastoma binding protein	X85133	oncogenes & tumor suppressors
E2F-3	D38550	cell cycle & growth regulators
E2F-5	U31556	cell cycle & growth regulators
E2F-related transcription factor	L23959	cell cycle & growth regulators
basic transcription factor 2 p44 (btf2p44) gene	U80017	cell cycle & growth regulators
transcription factor DP2 (Humdp2); E2F dimerization partner 2	U18422	cell cycle & growth regulators
growth factor receptor-bound protein 2 (GRB2) isoform	M96995	cell cycle & growth regulators
GRB-IR / GRB10	D86962	cell cycle & growth regulators
raf proto-oncogene serine/threonine-protein kinase (raf-1; c-raf)	X03484	cell cycle & growth regulators
b-raf	M95712	cell cycle & growth regulators
jun B transactivator	U20734	cell cycle & growth regulators
N-myc oncogene protein	Y00664	cell cycle & growth regulators
C-myc binding protein	D89667	cell cycle & growth regulators
p53-dependent cell growth regulator CGR19	U66469	apoptosis
apoptosis regulator bcl-2	M14745	apoptosis
Bcl2 and p53 binding protein Bbp/53BP2 (BBP/53BP2)	U09582	apoptosis
clone 53BP1 p53-binding protein mRNA, partial cds	U09477	apoptosis
apoptosis regulator bcl-w; KIAA0271	D87461	apoptosis
induced myeloid leukemia cell differentiation protein MCL-1	L08246	apoptosis
bcl-2-related protein A1; bfl-1 protein	U29680	apoptosis
BCL-2 homologous antagonist/killer (BAK) protein	U23765	apoptosis
brain-related apoptosis gene (BRAG-1); Bcl-2 homolog	AB011170	apoptosis

BAD protein (BCL-2 binding component 6)	U66879	apoptosis
BCL2/adenovirus E1B 19kD-interacting protein 2 (BNIP2) mRNA, complete cds	U15173	apoptosis
BCL2/adenovirus E1B 19kD-interacting protein 1 (BNIP1) mRNA, complete cds	AF083957	apoptosis
interleukin-1 beta convertase precursor (IL-1BC)	M87507	apoptosis
apopain precursor; cysteine protease CPP32; YAMA protein	U13737	apoptosis
ICH-2 protease precursor (EC 3.4.22.-); TX protease (ICEREL-II); caspase-4	U28014; U28015	apoptosis
cysteine protease MCH2 isoforms alpha and beta (MCH2); caspase-6 precursor (EC 3.4.22.-)	U20537	apoptosis
caspase-7 precursor (EC 3.4.22.-)	U37448	apoptosis
Apo-2 ligand; TNF-related apoptosis inducing ligand TRAIL	U37518	apoptosis
caspase-8 precursor (EC 3.4.22.-)	X98173	apoptosis
caspase-9 precursor (EC 3.4.22.-)	U56390	apoptosis
caspase-10 precursor; ICE-LIKE apoptotic protease 4	U60519	apoptosis
tyrosine-protein kinase receptor tyro3 precursor; tyrosine-protein kinase	D17517	oncogenes & tumor suppressors
TRAF5	AB000509	apoptosis
TRAF6	U78798	apoptosis
TRAF-interacting protein I-TRAF; TRAF family member-associated NF-kB activator TANK	U59863	apoptosis
TRAF-interacting protein (TRIP)	U77845	apoptosis
serine/threonine protein kinase NIK; binds specifically to TRAF2	Y10256	apoptosis
casper, a FADD- and caspase-related inducer of apoptosis (CASH-alpha + CASH-beta)	AF015450	apoptosis
cytotoxic ligand TRAIL receptor	AF016266	apoptosis
death domain containing protein CRADD	U79115	apoptosis
receptor interacting protein	U25994	apoptosis
DAXX; a FAS-binding protein that activates JNK and apoptosis	AF039136	apoptosis
tumor necrosis factor type 2 receptor associated protein (TRAP3)	U12597	apoptosis

CD40 receptor associated factor 1 (CRAF1)	U21092	apoptosis
inhibitor of apoptosis protein1 (HIAP-1) (C-IAP2)	U45878	apoptosis
inhibitor of apoptosis protein 2 (IAP-2)	U45879	apoptosis
TNF-alpha converting enzyme	U69611	apoptosis
ionizing radiation resistance-conferring protein; death-associated protein 3 (DAP-3)	U18321	apoptosis
Fas-activated serine/threonine (FAST) kinase	X86779	apoptosis
c-yes-1	M15990	oncogenes & tumor suppressors
FAS/APO 1	D49396	apoptosis
5'-AMP activated protein kinase, gamma1	U42412	oncogenes & tumor suppressors
Akt1; rac protein kinase alpha; protein kinase B; c-Akt	M63167	apoptosis
AKT2; rac protein kinase beta	M77198	apoptosis
seven in absentia homolog	U63295	apoptosis
signal transducer and activator of transcription 1-alpha/beta (STAT1)	M97935	oncogenes & tumor suppressors
apoptosis-related protein TFAR15 (TFAR15)	AF022385	apoptosis
signal transducer and transcription activator 5B (STAT5B)	U47686	oncogenes & tumor suppressors
CD27BP (Siva)	U82938	apoptosis
CSE1	AF053640	apoptosis
apoptosis inhibitor survivin	U75285	apoptosis
proto-oncogene rhoA multidrug resistance protein; GTP-binding protein (rhoA)	L25080	apoptosis
Pig7 (PIG7)	AF010312	apoptosis
Pig10 (PIG10)	AF010314	apoptosis
Pig11 (PIG11)	AF010315	apoptosis
Pig12 (PIG12)	AF010316	apoptosis
glutathione-S-transferase homolog	U90313	apoptosis
cdc42 homolog (G25K) (brain isoform + placental isoform)	U02570	apoptosis
macrophage colony stimulating factor I receptor precursor (CSF-1-R)	X03663	oncogenes & tumor suppressors
C-fos	V01512	oncogenes & tumor suppressors
c-kit	X06182	oncogenes & tumor suppressors

fgr proto-oncogene encoded p55-c-fgr protein	M19722	oncogenes & tumor suppressors
DNA mismatch repair protein MSH2	U03911	oncogenes & tumor suppressors
DNA mismatch repair protein MSH6 (mutS alpha 160-kDa subunit)	U54777	oncogenes & tumor suppressors
K-ras oncogene	M54968	oncogenes & tumor suppressors
MET	J02958	oncogenes & tumor suppressors
breast cancer susceptibility (BRCA2)	X95152	oncogenes & tumor suppressors
BRCA1-associated ring domain protein	U76638	oncogenes & tumor suppressors
p53 cellular tumor antigen	X54156	oncogenes & tumor suppressors
mdm2 protein; p53-associated protein	M92424	oncogenes & tumor suppressors
retinoblastoma susceptibility	L41870	oncogenes & tumor suppressors
RB2/p130	X74594	oncogenes & tumor suppressors
RBA/p48	X74262	oncogenes & tumor suppressors
RBP2 retinoblastoma binding protein	S66431	oncogenes & tumor suppressors
GADD153=growth arrest and DNA-damage-inducible	S40706	DNA damage response, repair & recombination
insulin-like growth factor I receptor (IGF1R)	X04434	receptors
DNA-PK catalytic subunit (XRCC7)	U47077	DNA damage response, repair & recombination
ataxia telangiectasia (ATM)	U82828	DNA damage response, repair & recombination
cation-independent mannose-6-phosphate receptor precursor (CI man-6-P receptor; CI-MPR)	Y00285	receptors
Ku protein subunit; ATP-dependent DNA helicase II 70-kDa subunit	M32865	DNA damage response, repair & recombination
Ku (p70/p80) subunit; ATP-dependent DNA helicase II 86-kDa subunit	M30938	DNA damage response, repair & recombination
DNA excision repair protein ERCC1	M13194	DNA damage response, repair & recombination
DNA ligase III (LIG3); polydeoxyribonucleotide synthase	X84740	DNA damage response, repair & recombination
DNA ligase IV; polydeoxyribonucleotide synthase (ATP)	X83441	DNA damage response, repair & recombination
DNA polymerase alpha-subunit	X06745	DNA damage response, repair & recombination
insulin-like growth factor binding protein 2 (IGFBP2)	X16302	receptors

recA-like protein HsRad51; DNA repair protein RAD51 homolog	L07493	DNA damage response, repair & recombination
DNA damage repair and recombination protein RAD52	U12134	DNA damage response, repair & recombination
DNA topoisomerase I (TOP1)	M60706	DNA damage response, repair & recombination
growth hormone-dependent insulin-like growth factor-binding protein	M35878	receptors
IGFBP5	L27560	receptors
DNA excision repair protein ERCC2 3' end; DNA-repair protein complementing XP-D cells	X52222	DNA damage response, repair & recombination
IGFBP6	M62402	receptors
ERCC5 excision repair protein	X69978	DNA damage response, repair & recombination
6-O-methylguanine-DNA methyltransferase (MGMT); methylated-DNA-protein-cysteine methyltransferase	M29971	DNA damage response, repair & recombination
muscle-specific DNase I-like (DNase X)	X90392	DNA damage response, repair & recombination
DNA mismatch repair protein hmlh1	U07418	DNA damage response, repair & recombination
GTP-binding protein ras associated with diabetes (RAD1)	L24564	DNA damage response, repair & recombination
replication factor C 37-kDa subunit	M87339	DNA damage response, repair & recombination
replication factor C 38-kDa subunit (RFC38); activator 1 38-kDa subunit	L07541	DNA damage response, repair & recombination
replication protein A 70-kDa subunit (RP-A) (RF-A); single-stranded DNA-binding protein	M63488	DNA damage response, repair & recombination
superoxide dismutase 1 cytosolic	X02317	DNA damage response, repair & recombination
single-stranded DNA-binding protein pur-alpha	M96684	DNA damage response, repair & recombination
HHR6A (yeast RAD 6 homolog)	M74524	DNA damage response, repair & recombination
lysozyme	M19045	DNA damage response, repair & recombination
Notch2 Notch homolog 3	U97669	cell fate & development regulators
CDW40 antigen; CD40L receptor precursor	X60592	receptors

Jagged 1	AF028593	cell fate & development regulators
Jagged 2	AF029778	cell fate & development regulators
delta-like protein precursor (dlk); contains fetal antigen 1 (FA1) (DLK)	U15979	cell fate & development regulators
lunatic fringe	U94354	cell fate & development regulators
wnt-2 protein precursor; IRP protein; int-1 related protein	X07876	cell fate & development regulators
Wnt-5a	L20861	cell fate & development regulators
frizzled-related FrzB (Fritz); frezzled (fre)	U24163	cell fate & development regulators
dishevelled 2 (DVL)	AF006012	cell fate & development regulators
patched homolog (PTC)	U43148	cell fate & development regulators
smoothened	U84401	cell fate & development regulators
retinoic acid receptor epsilon (RAR-epsilon); retinoic acid receptor beta2 (RAR-beta2)	Y00291	receptors
tumor necrosis factor type 1 receptor associated protein (TRAP1) mRNA, partial cds	U12595	receptors
Tumor necrosis factor receptor 2 (75kD) (TNFR2)	U52165	receptors
epidermal growth factor receptor substrate 15 (EPS15); AF-1P protein	U07707	receptors
epidermal growth factor receptor kinase substrate EPS8	U12535	receptors
erythropoietin receptor (EPOR)	M60459	receptors
NT-3 growth factor receptor precursor; trk-c tyrosine kinase; GP145-TRKC	U05012	receptors
GARP	Z24680	receptors
retinoic acid receptor alpha (RXRA)	X52773	receptors
HGF activator like	D49742	receptors
N-sam; fibroblast growth factor receptor1 precursor (FGFR1)	X66945	receptors

low-affinity nerve growth factor receptor (NGF receptor; NGFR); GP80-LNGFR	M14764	receptors
platelet-derived growth factor receptor alpha (PDGFRA); CD140A antigen	M21574	receptors
beta platelet-derived growth factor receptor precursor (PDGFR-beta); CD140B antigen	J03278	receptors
colon carcinoma kinase-4 (CCK4); transmembrane receptor precursor (PTK7)	U33635	receptors
retinoic acid receptor gamma	M38258	receptors
transforming growth factor (TGF)-beta receptor type III precursor (TGFR-3); betaglycan	L07594	receptors
transmembrane protein TMP21	AJ004913	receptors
high-affinity nerve growth factor receptor precursor	X03541	receptors
brain-derived neurotrophic factor (BDNF)/NT-3 growth factors receptor precursor	U12140	receptors
hemopoietic progenitor cell CD34 antigen precursor	S53910	cell adhesion, motility & invasion
CD59	M84349	cell adhesion, motility & invasion
angiopoietin 1 receptor precursor; tyrosine-protein kinase receptor TIE-2	L06139	angiogenesis regulators
collagen type I	J03464	cell adhesion, motility & invasion
collagen type II alpha-1	X16468	cell adhesion, motility & invasion
collagen type III pro-alpha-1	X14420	cell adhesion, motility & invasion
collagen type IV alpha	M26576	cell adhesion, motility & invasion
collagen type VI alpha-3	X52022	cell adhesion, motility & invasion
collagen type VIII alpha-1	X57527	cell adhesion, motility & invasion
vascular endothelial growth factor B precursor (VEGF-B)	U43368	angiogenesis regulators

collagen type XI pro-alpha-2	U32169	cell adhesion, motility & invasion
collagen type XVI alpha-1	M92642	cell adhesion, motility & invasion
collagen type XVIII alpha	L22548	cell adhesion, motility & invasion
laminin alpha-4 subunit precursor (LAMA4)	S78569	cell adhesion, motility & invasion
laminin beta-2 subunit precursor (LAMB2); S-laminin	M94362	cell adhesion, motility & invasion
laminin beta-1 subunit precursor (LAMB1); laminin B1 chain	M61916	cell adhesion, motility & invasion
laminin gamma-1 subunit precursor (LAMG1); laminin B2 chain	M55210	cell adhesion, motility & invasion
laminin 67kDa RECEPTOR	X15005	cell adhesion, motility & invasion
nidogen precursor (NID); entactin	M30269	cell adhesion, motility & invasion
tenascin precursor (TN); hexabrachion; cytotactin; neuronectin	X78565	cell adhesion, motility & invasion
versican core protein precursor; large fibroblast proteoglycan	J02814	cell adhesion, motility & invasion
sparc precursor (secreted protein acidic and rich in cysteine; osteonectin) (ON)	J03040	cell adhesion, motility & invasion
thrombospondin 1 precursor	X14787	cell adhesion, motility & invasion
thrombospondin 2 precursor	L12350	cell adhesion, motility & invasion
vitronectin precursor; serum spreading factor; S-protein (contains somatomedin B)	X03168	cell adhesion, motility & invasion
fibronectin precursor (FN)	X02761	cell adhesion, motility & invasion
heparan sulfate proteoglycan (HSPG2)	M85289	cell adhesion, motility & invasion
integrin alpha subunit	X68742	cell adhesion, motility & invasion
vascular endothelial growth factor C precursor (VEGF-C)	U43142	angiogenesis regulators
integrin alpha-3 chain	M59911	cell adhesion, motility & invasion

integrin alpha-4 subunit precursor (integrin alpha-IV; ITGA4); VLA-4; CD49D antigen	L12002	cell adhesion, motility & invasion
placenta growth factors 1 (PLGF-1)	X54936	angiogenesis regulators
integrin alpha 7B	X74295	cell adhesion, motility & invasion
integrin alpha9	D25303	cell adhesion, motility & invasion
integrin alpha-E precursor (ITGAE); mucosal lymphocyte-1 antigen; hml-1 antigen; CD103 antigen	L25851	cell adhesion, motility & invasion
integrin beta1	M34189	cell adhesion, motility & invasion
integrin beta 4	X53587	cell adhesion, motility & invasion
integrin beta-5 subunit (ITGB5)	J05633	cell adhesion, motility & invasion
integrin beta8	M73780	cell adhesion, motility & invasion
focal adhesion kinase (FADK); proline-rich tyrosine kinase 2 (PYK2)	L13616	cell adhesion, motility & invasion
integrin-linked kinase (ILK)	U40282	cell adhesion, motility & invasion
cell adhesion kinase beta (CAKbeta); protein tyrosine kinase Pyk2	U43522	cell adhesion, motility & invasion
paxillin	U14588	cell adhesion, motility & invasion
alpha 1,2-mannosidase 1B mRNA	AF027156	cell adhesion, motility & invasion
zyxin related protein ZRP-1	AF000974	cell adhesion, motility & invasion
beta 3-endonexin	U37139	cell adhesion, motility & invasion
cytohesin-1; Sec7p-like protein	U70728	cell adhesion, motility & invasion
CD9 antigen; p24; leukocyte antigen MIC3; motility-related protein (MRP-1)	M38690	cell adhesion, motility & invasion
ezrin (cytovillin 2)	X51521	cell adhesion, motility & invasion
moesin-ezrin-radixin-like protein; merlin; schwannomin; neurofibromatosis 2	L11353	cell adhesion, motility & invasion
neural cell adhesion molecule L1 precursor (N-CAM L1); MIC5	AF002246	cell adhesion, motility & invasion

ninjurin-1	U91512	cell adhesion, motility & invasion
formyl peptide receptor 1	M60626	cell adhesion, motility & invasion
P37NB	U32907	cell adhesion, motility & invasion
semaphorin (CD100)	U60800	cell adhesion, motility & invasion
semaphorin E	AB000220	cell adhesion, motility & invasion
TAX1; axonin-1/TAQ1	X67734	cell adhesion, motility & invasion
leukocyte antigen related protein precursor (LAR); PTPRF	Y00815	cell adhesion, motility & invasion
hyaluronan receptor (RHAMM)	U29343	cell adhesion, motility & invasion
platelet glycoprotein IV (GPIV) (GPIIIB; CD36 antigen) (PAS IV); PAS-4 protein	M98399	cell adhesion, motility & invasion
caveolin-2	AF035752	cell adhesion, motility & invasion
FGFR3; FLG-2	M64347	angiogenesis regulators
keratinocyte growth factor receptor (KGFR); fibroblast growth factor receptor 2 (FGFR2)	M80634	angiogenesis regulators
MMP-1; collagenase-1	X54925	invasion regulators
MMP-2; gelatinase A	Z48482	invasion regulators
MMP-16	D85511	invasion regulators
MMP-7; matrilysin	X07819	invasion regulators
EB1 (protein that binds to APC)	U51677	cell -cell interactions
MMP-10; stromelysin-2	X07820	invasion regulators
MMP-13; collagenase-3	X75308	invasion regulators
protocadherin 43	L11373	cell -cell interactions
desmoplakin I	M77830	cell -cell interactions
envoplakin (EVPL)	U53786	cell -cell interactions
bullous pemphigoid antigen	M69225	cell -cell interactions
TIMP-2 (MI)	J05593	invasion regulators
TIMP-3; mitogen-inducible gene 5 (mig-5)	Z30183	invasion regulators
basigin precursor (BSG); leukocyte activation antigen M6	X64364	invasion regulators

urokinase-type plasminogen activator precursor (EC 3.4.21.73); U-plasminogen activator (UPA)	X02419	invasion regulators
tissue-type plasminogen activator precursor (EC 3.4.21.68); T-plasminogen activator (T-PA)	M15518	invasion regulators
plasminogen precursor (EC 3.4.21.7)	M34276	invasion regulators
placental plasminogen activator inhibitor-2 (PAI-2); monocyte ARG-serpin; urokinase inhibitor; PLANH2	Y00630	invasion regulators
protein C inhibitor; plasma serine protease inhibitor precursor; plasminogen activator inhibitor-3 (PAI3)	M68516	invasion regulators
urokinase-type plasminogen activator receptor	U09937	invasion regulators
low-density lipoprotein receptor-related protein 1 precursor (LRP); alpha-2-macroglobulin receptor (A2MR)	X13916	invasion regulators
alpha-2-macroglobulin precursor (alpha-2-M)	M11313	invasion regulators
platelet basic protein precursor (PBP)	M54995	invasion regulators
alpha-2-macroglobulin receptor-associated protein precursor (alpha-2-MRAP)	M63959	invasion regulators
nucleoside diphosphate kinase A (EC 2.7.4.6) (NDK A)	X17620	invasion regulators
c-myc purine-binding transcription factor puf; nucleoside diphosphate kinase B (NDP kinase B; NDK B)	M36981	invasion regulators
nm23-H4; nucleoside-diphosphate kinase (EC 2.7.4.6); nucleoside 5'-diphosphate phosphotransferase (NDK)	Y07604	invasion regulators
malignant melanoma metastasis-suppressor (KiSS-1) gene	U43527	invasion regulators
metastasis-associated MTA1	U35113	invasion regulators
metalloprotease/disintegrin/cysteine-rich protein precursor (MDC9)	U41766	invasion regulators
DDX8; RNA helicase	D50487	invasion regulators
forkhead-like 7	AF048693	Rho family small GTPases & their regulator
rhoG	X61587	Rho family small GTPases & their regulator

Rho6 protein	Y07923	Rho family small GTPases & their regulator
Rho8 protein	X95282	Rho family small GTPases & their regulator
ephrin-B3 precursor; eph-related receptor tyrosine kinase ligand 8 (LERK-8)	U66406	cell -cell interactions
ras-like protein TC10	M31470	Rho family small GTPases & their regulator
ras-like small GTPase TTF	Z35227	Rho family small GTPases & their regulator
rhoHP1	D85815	Rho family small GTPases & their regulator
rho-associated coiled-coil containing protein kinase p160ROCK	U43195	Rho family small GTPases & their regulator
CDC42 GTPase-activating protein	U02570	Rho family small GTPases & their regulator
T-lymphoma invasion and metastasis inducing TIAM1	U16296	Rho family small GTPases & their regulator
rho/rac guanine nucleotide exchange factor (rho/rac GEF); faciogenital dysplasia protein (FGD1)	U64105	Rho family small GTPases & their regulator
ephrin type-A receptor 2 precursor; epithelial cell kinase (ECK); tyrosine-protein kinase receptor ECK	M59371	cell -cell interactions
rho GDP dissociation inihibitor 2 (rho GDI 2); LY-GDI	L20688	Rho family small GTPases & their regulator
rho GDP dissociation inihibitor 1 (rho GDI 1)	X69550	Rho family small GTPases & their regulator
p21-activated kinase; p65-PAK; serine/threonine-protein kinase PAK-alpha	U24152	Rho family small GTPases & their regulator
neural-cadherin precursor (N-cadherin); cadherin-2	S42303	cell -cell interactions
cadherin-3 placental-cadherin precursor; P-cadherin	X63629	cell -cell interactions
cadherin-5 vascular endothelial-cadherin precursor; VE-cadherin; 7B4 antigen; CD144 antigen	X79981	cell -cell interactions
cadherin-6	D31784	cell -cell interactions
cadherin-8	L34060	cell -cell interactions
casein kinase II, alpha subunit	J02853	cell -cell interactions

ephrin type-B receptor 2 precursor; tyrosine-protein kinase receptor EPH-3; DRT; HEK; ERK	L41939	cell -cell interactions
cadherin-13 T-cadherin precursor (truncated-cadherin); H-cadherin; heart-cadherin	U59289	cell -cell interactions
cadherin-14 muscle-cadherin precursor; M-cadherin; cadherin-14; cadherin-15	U59325	cell -cell interactions
alpha-catenin; cadherin-associated protein; alpha E-catenin	D13866	cell -cell interactions
alpha-catenin related protein (catenin alpha-2)	M94151	cell -cell interactions
beta-catenin	X87838	cell -cell interactions
tyrosine-protein kinase HCK (EC 2.7.1.112); P59-HCK & P60-HCK; hemopoietic cell kinase	M16591	cell -cell interactions
APC	M73548	cell -cell interactions
Tumor necrosis factor member2 (TNF)	X02910	growth factors & cytokines
amphiregulin (AR); colorectum cell-derived growth factor (CRDGF)	M30704	growth factors & cytokines
brain-derived neurotrophic factor (BDNF)	M61176	growth factors & cytokines
beta NGF	X52599	growth factors & cytokines
clone pSK1 interferon gamma receptor accessory factor-1 (AF-1); interferon-gamma receptor beta chain	U05875	growth factors & cytokines
BIGH3	M77349	growth factors & cytokines
bone morphogenetic protein 1 (BMP1)	U50330	growth factors & cytokines
interferon-alpha/beta receptor alpha subunit precursor (IFN-alpha receptor; IFNAR)	J03171	growth factors & cytokines
bone morphogenetic protein 3B	D49493	growth factors & cytokines
bone morphogenetic protein 2B (BMP2B)	D30751	growth factors & cytokines
bone morphogenetic protein 6	M60315	growth factors & cytokines
bone morphogenetic protein 7; osteogenic protein 1	X51801	growth factors & cytokines
bone morphogenetic protein 8; osteogenic protein 2	M97016	growth factors & cytokines
BPGF-1	L42379	growth factors & cytokines

connective tissue growth factor (CTGF)	M92934	growth factors & cytokines
heparin-binding EGF-like growth factor (HBEGF); diphtheria toxin receptor (DTR)	M60278	growth factors & cytokines
interferon-alpha/beta receptor beta subunit precursor (IFN-alpha-R)	L42243	growth factors & cytokines
fibrillin 2 (congenital contractural arachnodactyly)	U03272	growth factors & cytokines
FGF2; heparin-binding growth factor 2 precursor; prostatropin	J04513	growth factors & cytokines
keratinocyte growth factor (KGF); fibroblast growth factor 7 (FGF-7)	M60828	growth factors & cytokines
cytokine humig; interferon-gamma-induced monokine (MIG)	X72755	growth factors & cytokines
glia maturation factor beta (GMF-beta)	M86492	growth factors & cytokines
glial growth factor precursor (GGFHPP2); neuregulin; heregulin-beta1	L12261	growth factors & cytokines
transforming growth factor beta2 precursor (TGF-beta2; TGFB2)	M19154	growth factors & cytokines
interferon-gamma induced protein precursor (gamma-IP10)	X02530	growth factors & cytokines
transcription factor ETR103; early growth response protein 1 (EGR-1) (KROX24)	X52541	growth factors & cytokines
hepatocyte growth factor-like protein; macrophage-stimulating protein (MSP)	L11924	growth factors & cytokines
hepatoma-derived growth factor (HDGF)	D16431	growth factors & cytokines
hepatocyte growth factor (HGF); scatter factor (SF); hepatopoeitin A	X16323	growth factors & cytokines
interleukin-1 receptor antagonist protein precursor (IL-1RA; IRAP)	U65590	growth factors & cytokines
interleukin-1 alpha precursor (IL-1 alpha; IL1A); hematopoietin-1	M28983	growth factors & cytokines
interleukin-1 beta precursor (IL-1 ; IL1B); catabolin	K02770	growth factors & cytokines
MADER	X70991	growth factors & cytokines
interleukin-6 precursor (IL-6); B-cell stimulatory factor 2 (BSF-2)	X04430	growth factors & cytokines
interleukin-15 (IL-15)	U14407	growth factors & cytokines
interferon gamma precursor (IFN-gamma; IFNG); immune interferon	V00543	growth factors & cytokines

leukocyte interferon-inducible peptide	X57351	growth factors & cytokines
leukemia inhibitory factor precursor (LIF); differentiation-stimulating factor (D factor)	X13967	growth factors & cytokines
PDGF associated protein	U41745	growth factors & cytokines
platelet-derived growth factor A subunit precursor (PDGFA; PDGF-1)	X06374	growth factors & cytokines
platelet-derived growth factor B subunit precursor (PDGFB; PDGF2); bacaplermin; c-sis	X02811	growth factors & cytokines
stromal cell derived factor 1 precursor (SDF1); pre B-cell growth stimulating factor (PBSF)	L36033	growth factors & cytokines
TGF-b superfamily receptor type I (ALK-1) (SRK3)	L17075	growth factors & cytokines
transforming growth factor-beta 3 (TGF-beta3)	X14885	growth factors & cytokines
thrombopoietin precursor (THPO); megakaryocyte colony stimulating factor	L33410	growth factors & cytokines
transforming growth factor-alpha (TGF-alpha; TGFA); EGF-like TGF (ETGF)	X70340	growth factors & cytokines
interferon-stimulated transcription factor 3, gamma (48kD)	M87503	growth factors & cytokines
ubiquitin	S79522	housekeeping gene
phospholipase A2	U03090	housekeeping gene
adenine phosphoribosyltransferase (APRT)	Y00486	housekeeping gene
tubulin alpha	L11645	housekeeping gene
HLA class I histocompatibility antigen A-3 alpha chain (MHC)	D32129	housekeeping gene
aortic-type smooth muscle alpha-actin gene, exon 9	3216M3	housekeeping gene
ribosomal protein S5	U14970	housekeeping gene
p300/CBP	U47741	nuclear receptor or nuclear receptor transcriptional coupling
SRC-1	U40396	nuclear receptor or nuclear receptor transcriptional coupling
N-CoR/SMRT	AF044209/ U37146	nuclear receptor or nuclear receptor transcriptional coupling
ACTR	AF036892	nuclear receptor or nuclear receptor transcriptional coupling

RIP140	X84373	nuclear receptor or nuclear receptor transcriptional coupling
TRIP1	L38810	nuclear receptor or nuclear receptor transcriptional coupling
TIF2	X97674	nuclear receptor or nuclear receptor transcriptional coupling
Smad3	AB004924	nuclear receptor or nuclear receptor transcriptional coupling
efp	D21205	nuclear receptor or nuclear receptor transcriptional coupling
lactoferrin	X53961	nuclear receptor or nuclear receptor transcriptional coupling
progesteron receptor	M15716	nuclear receptor or nuclear receptor transcriptional coupling
cathepsin G	J04990	nuclear receptor or nuclear receptor transcriptional coupling
pS2 protein	X52003	nuclear receptor or nuclear receptor transcriptional coupling
prolactin	E02152	nuclear receptor or nuclear receptor transcriptional coupling
ARA70	L49399	nuclear receptor or nuclear receptor transcriptional coupling
vitamin D receptor	J03258	nuclear receptor or nuclear receptor transcriptional coupling
p38	L35253	kinase-type signal transduction
p38 gamma	U66243	kinase-type signal transduction
JNK1	L26318	kinase-type signal transduction
JNK2	U09759	kinase-type signal transduction
JNK3	AA992006	kinase-type signal transduction
ERK1	M76585	kinase-type signal transduction
BMKa,b,g	U29725-U29727	kinase-type signal transduction
DAX1	U31929	gonad differentiation
SOX9	Z46629	gonad differentiation

WT1	X51630	gonad differentiation
SRY	L10101	gonad differentiation
Ad4BP/SF-1	D84206-D84209	gonad differentiation
EMX2	X68880	gonad differentiation
c-Fos	K00650/M16287	oncogenes & tumor suppressors
c-Myc	J00120/K01908	oncogenes & tumor suppressors
Bcl-2	M13994-M13995	oncogenes & tumor suppressors
Bax a,b,g	L22473-L22475	oncogenes & tumor suppressors
Bax d	U19599	oncogenes & tumor suppressors
Bcl-x	U72398	oncogenes & tumor suppressors
NGF receptor	M14764	receptor-type kinase
FGF receptor	M34641	receptor-type kinase
VEGF receptor	AF016050	receptor-type kinase
PDGF receptor	M21616	receptor-type kinase
CSF1 receptor	M33208-M33210	receptor-type kinase
EGF receptor	M29366	receptor-type kinase
insulin receptor	M10051	receptor-type kinase

The genes that are potentially influenced by endocrine disruptors are further exemplified by the gene

for estrogen receptor, which is known to bind diethylstilbestrol, bisphenol-A, 17 β-estradiol and the like, as well as genes involved in the signal transduction pathway for the estrogen receptor.

A gene that is influenced by an endocrine disruptor can be detected as follows.

As used herein, a DNA array refers to a support onto which a gene or a DNA fragment derived from the gene is immobilized and includes, for example, a so-called DNA chip. Any supports which can be used for hybridization may be used. A slide glass, a silicone chip, a nitrocellulose or nylon membrane or the like is usually used. For example, the gene or a DNA fragment thereof to be immobilized onto the support can be prepared as follows. A primer pair for PCR amplification which is optimal for the method of the present invention can be prepared based on a base sequence identified by a GenBank accession no. assigned to a gene to be immobilized or the product of the gene using a primer analysis/construction software such as Oligo™ Primer Analysis Software (Takara Shuzo). A PCR-amplified fragment of interest can be obtained by using the primer pair and a genomic DNA, a genomic DNA library or a cDNA library as a template according to a standard protocol attached to a commercially available PCR kit. The resulting DNA fragment can be purified using, for example, Microcon-100 (Takara

Shuzo). The purified DNA fragment can be preferably used in the method of the present invention. Furthermore, a DNA array can be prepared by immobilizing the gene or a fragment thereof onto a support according to a known method, for example, by introducing an amino group to the support. Also, a DNA array onto which gene are arrayed and immobilized at high density can be prepared by conducting the immobilization procedure using an instrument for preparing DNA arrays such as an instrument for preparing DNA chips from GMS.

C3 cont.)

The use of such a DNA array makes it possible to simultaneously measure the contents of various nucleic acid molecules in a nucleic acid sample and has the advantage that the measurement can be conducted using a small amount of a nucleic acid sample.

Any genes or DNA fragments derived from the genes are immobilized onto the DNA array used in the present invention. Preferably, genes encoding proteins that are expected to have functions related to endocrine disrupting activities or DNA fragments derived from the genes are immobilized. If a fragment is used, a fragment of, for example, about 1 kb in length can be preferably used, although the length of the fragment is not limited to specific one. The length may be shorter or longer than that described above as long as the fragment specifically

hybridizes with a nucleic acid from a test sample. Examples of such genes include, but are not limited to, a gene for a hormone receptor, a gene encoding a cofactor for a receptor, a gene encoding a protein involved in signal transduction from a receptor, a gene encoding a protein involved in biosynthesis or metabolism of a hormone and an oncogene.

For example, mRNAs prepared from a cell or a tissue (organ) that is sensitive to an endocrine disruptor or cDNAs obtained by reverse transcription using the mRNAs as templates can be used as nucleic acid samples containing a gene of which the expression is altered as a result of the influence of the endocrine disruptor. Such mRNAs are obtained over time or on different days after being exposed to the endocrine disruptor.

The cell to be exposed to a sample containing an endocrine disruptor may be a cell collected from an organism, or it may be a cultured cell. The tissue is not limited to specific one as long as it is supposed to be influenced by an endocrine disruptor. Furthermore, the origin of the cell or the tissue, or the organism to be used is not limited to human. The length of the time of exposure to an endocrine disruptor may vary depending on the organism, the endocrine disruptor, the gene that is influenced by the endocrine disruptor or the like to be

used.

On the other hand, a nucleic acid sample containing mRNAs, or cDNAs therefor, similarly prepared from a cell, a tissue or an organism as a control is subjected to hybridization under stringent conditions. The nucleic acid sample can be suitably labeled as follows such that it can be readily determined whether or not the nucleic acid sample hybridizes with a DNA on a DNA array.

For example, a radioisotope, a fluorescent substance, chemiluminescent substance, an antigen recognized by an appropriate antibody or the like can be used for labeling. Alternatively, hybridization may be first conducted without labeling the nucleic acid sample, and then an intercalating substance that emits fluorescence or chemiluminescence may be used for labeling.

Hybridization of the thus obtained nucleic acid sample with the DNA on the DNA array can be conducted according to a known method. It is natural to conduct hybridization and washing steps under optimal conditions depending on the length of the DNA on the DNA array or the like. These steps can be conducted under conditions, for example, as described in Molecular Cloning, A Laboratory Manual, 2nd ed., pp. 9.52-9.55 (1989).

By comparing results of hybridization for a control nucleic acid sample with those for a nucleic acid

sample derived from a cell, a tissue or an organism which has been exposed to a sample containing an endocrine disruptor, a gene of which the signal intensity is significantly different among the two nucleic acid samples can be detected. Specifically, an array is subjected to hybridization with a nucleic acid sample labeled as described above. A signal intensity of radioactivity, fluorescence, luminescence or the like for the hybridized array is detected using a specialized measuring instrument such as a chromatogram scanner or image analyzer. A gene of which the expression is significantly altered as a result of the influence of an endocrine disruptor can be detected based on the difference in the signal intensity.

The gene expression for a control nucleic acid sample can be compared on the same DNA array with that for a nucleic acid sample derived from a cell, a tissue or an organism that has been exposed to an endocrine disruptor on the same DNA array by using a multiple wavelength detecting fluorescence analyzer which is capable of detecting plural labels (e.g., two types of fluorescence). For example, a nucleic acid sample derived from a cell which has been exposed to an endocrine disruptor is fluorescence-labeled with Cy3-dUTP, whereas a control nucleic acid sample is fluorescence-labeled with Cy5-dUTP. The difference in gene expression between the two nucleic acid samples can be

detected as difference in color by mixing equal amounts of the nucleic acid samples and subjecting the mixture to hybridization with a DNA array. A gene of which the expression level is significantly altered as a result of the influence of the endocrine disruptor can be detected based on the results.

The gene is also useful as an index for detecting an endocrine disruptor.

A gene that is influenced by an endocrine disruptor is selected by comparing a signal intensity detected as an index of expression level with that obtained using a nucleic acid sample prepared using a control sample. For example, a value is calculated by dividing a fluorescence signal value for a sample containing an endocrine disruptor by a fluorescence signal value for a control sample. A value greater than 1.00 indicates that the gene expression is promoted by the treatment with the test substance. A value smaller than 1.00 indicates that the gene expression is suppressed by the treatment with the test substance. A value equal to 1.00 indicates that the gene is not influenced by the treatment with the test substance. If the expression is promoted, the value is greater than 1.10, preferably 1.30, more preferably 2.00. If the expression is suppressed, the value is smaller than 0.90, preferably 0.80, more preferably 0.70.

As described above, the expression of genes that are influenced by endocrine disruptors (for example, a gene for a nuclear receptor in a cell and a number of genes involved in the downstream signal transduction pathway) can be detected simultaneously, in vitro, rapidly and with high sensitivity according to the method of the present invention. In addition, it is possible to find involvement of a known gene in a previously unknown signal transduction pathway.

(2) An endocrine disruptor can be detected using the expression of a gene that is influenced by the endocrine disruptor as an index as follows.

A DNA array, onto which a gene which has been confirmed to be influenced by an endocrine disruptor is immobilized as described above, is prepared.

A nucleic acid sample is prepared from a cell, a tissue or an organism which is suspected to be influenced by the endocrine disruptor as described above. The nucleic acid sample is then hybridized as described above. Change in gene expression can be determined based on the difference between the signal intensities. The presence of the endocrine disruptor can be determined based on the results.

In another embodiment, the presence of an endocrine disruptor can be also determined as follows. An

RNA or a DNA that competes with an mRNA for a gene that has been confirmed to be influenced by the endocrine disruptor is prepared. A competitive RT-PCR is conducted using the RNA or the DNA as an internal standard. The expression level of the influenced gene is then quantitatively determined by the competitive RT-PCR.

As described above, the presence or the absence of an endocrine disruptor can be substantially and readily judged using the expression of a gene that is influenced by a endocrine disruptor (for example, a gene for a nuclear receptor in a cell or one of a number of downstream genes) as an index according to the method of the present invention.

(3) A substance that potentially causes endocrine disruption can be detected as follows.

As used herein, a substance that potentially causes endocrine disruption means a substance that potentially influences the normal activity of a hormone which is naturally exerted in a living body. Substances of which the activities have been already confirmed and substances of which the activities have not been confirmed yet are included within the definition.

A DNA array for detecting a substance that potentially causes endocrine disruption is prepared by immobilizing in a manner as described above a gene which

has been confirmed to be influenced by an endocrine disruptor according to a method as described in (1) above.

A nucleic acid sample is prepared from a cell, a tissue or an organism which has been exposed to a sample that is suspected to contain a substance that potentially causes endocrine disruption as described above. The nucleic acid sample is then hybridized as described above. Change in gene expression can be determined based on the difference between the signal intensities. The substance can be judged as an endocrine disruptor based on the results.

A substance can be considered to be an endocrine disruptor based on the results not only in case where changes in signals are observed for all of the DNAs on the DNA array but also in case where the changes are observed for a portion of the DNAs. In particular, if the changes in signal strength are observed for a portion of the DNAs, the detection method can be optimized by further selecting the genes that are influenced by the substance action according to the method as described in (1) above such that a substance that causes endocrine disruption as the substance does can be detected more exactly.

In another embodiment, an RNA or a DNA that competes with an mRNA for a gene that has been confirmed to be influenced by the endocrine disruptor as described above

is prepared. A competitive RT-PCR is conducted using the RNA or the DNA as an internal standard. The degree of endocrine disruption can be quantitatively detected based on the expression of the gene. Any methods can be preferably used for detecting, or quantifying the expression of, a gene that is influenced by an endocrine disruptor obtained as described in (1) above as long as the methods can be used for the detection/quantification of the gene.

(4) The DNA array of the present invention

As used herein, a DNA array refers to a support onto which a gene or a fragment thereof is immobilized and includes, for example, a so-called DNA chip.

Any supports which can be used for hybridization may be used for the DNA array of the present invention. Usually, a slide glass, a silicone chip, a nitrocellulose or nylon membrane or the like is used. Preferably, a support made from a material which is non-porous and has a smooth surface (e.g., a glass such as a slide glass) can be preferably used. Any supports having surfaces onto which DNAs can be immobilized through covalent bonds or non-covalent bonds can be used. Supports having hydrophilic or hydrophobic functional groups on their surfaces are preferably used. Examples of the preferable functional groups on the surfaces of the supports include, but are not

limited to, a hydroxy group, an amino group, a thiol group, an aldehyde group, a carboxyl group and an acyl group. The functional group may be present as a surface property of the support itself, or it may be introduced by surface treatment. Examples of supports with surface treatment include a glass treated with a commercially available silane coupling agent such as aminoalkylsilane or treated with a polycation such as polylysine or polyethyleneimine. Several treated slide glasses are commercially available.

A gene that is influenced by an endocrine disruptor or a DNA fragment derived from the gene is immobilized onto the DNA array of the present invention. The DNA array may be a DNA microarray in which double-stranded DNAs of the genes or the DNA fragments derived from the genes are arrayed and immobilized onto the same support under denaturing conditions. At least a portion of the immobilized DNA may be single-stranded. The DNA array herein may be prepared by spotting double-stranded DNAs onto the same support in array under denaturing conditions. The density of the array in the DNA array of the present invention is not specifically limited. For example, a DNA array with high density such as an array onto which DNAs are immobilized at 100 dot/cm^2 or more can be preferably used.

The DNA fragment to be arrayed and immobilized

onto a support in the present invention is not limited to specific one. In general, a double-stranded polynucleotide of 50 bases or more in length or a derivative thereof, which is prepared by enzymatic amplification by polymerase chain reaction (PCR) or the like and converted into single-stranded DNAs or derivatives thereof by denaturing upon immobilization onto a support for immobilizing a DNA, can be preferably used. The derivative may have modification which enables the immobilization onto the surface of the support. Examples of the derivatives include, but are not limited to, a DNA into which a functional group such as an amino group or a thiol group is introduced at the 5'-terminus of the DNA.

For example, a DNA amplified by PCR or the like using a genomic DNA library or a cDNA library as a template can be used as a gene or a DNA fragment to be immobilized onto a support. An oligonucleotide synthesized based on a known nucleotide sequence can also be used. A nucleic acid other than a DNA which is known in the art to be able to be used for hybridization (for example, an RNA prepared by in vitro transcription) can be immobilized in place of a DNA. The DNA array can be prepared by immobilizing the gene or a fragment thereof onto a support according to a known method, for example, by introducing an amino group to the support. The DNA array of the present invention onto which genes are

arrayed and immobilized can be prepared by conducting the immobilization procedure using an instrument for preparing DNA arrays such as an instrument for preparing DNA chips from GMS.

A gene encoding a protein having a function involved in the exertion of an activity of a hormone, or a fragment thereof, is immobilized onto the DNA array used in the present invention. If a fragment is used, a fragment of, for example, about 100 b to about 1 kb in length can be preferably used, although the length of the fragment is not limited to specific one. The length may be shorter or longer than that described above as long as the fragment specifically hybridizes with a nucleic acid from a test sample. Examples of such genes include, but are not limited to, a gene for a hormone receptor, a gene encoding a cofactor for a receptor, a gene encoding a protein related to signal transduction from a receptor, a gene encoding a protein related to biosynthesis or metabolism of a hormone, an oncogene and the like. In addition, a gene that is influenced by an endocrine disruptor, for example, obtained according to the method as described in (1) above may be immobilized. Furthermore, since all of the genes that are influenced by endocrine disruptors can be obtained according to the method as described in (1) above, a DNA array for detecting genes that are influenced by endocrine

disruptors onto which all of such genes are immobilized can be prepared.

As described above, for example, a gene for a nuclear receptor in a cell and genes related to the downstream signal transduction pathway can be detected in vitro, rapidly and with high sensitivity by using the method and the DNA array of the present invention. Thus, the presence or the absence of a substance that potentially causes endocrine disruption can be substantially and readily judged.

The following examples further illustrate the present invention in detail but are not to be construed to limit the scope thereof.

Example 1

Genes that are potentially influenced by endocrine disruptors are shown in Table 3.

Table 3

Agent (Gene product)	GenBank accession no.
1. Nuclear receptor or nuclear receptor transcriptional coupling	
p300/CBP	U47741
SRC-1	U40396
N-CoR/SMRT	AF044209/U37146
ACTR	AF036892
RIP140	X84373
TRIP1	L38810
TIF2	X97674
Smad3	AB004924
efp	D21205
lactoferrin	X53961
progesteron receptor	M15716
cathepsin G	J04990
pS2 protein	X52003
prolactin	E02152
ARA70	L49399
vitamin D receptor	J03258
2. Kinase-type signal transduction	
p38	L35253
p38 gamma	U66243
JNK1	L26318
JNK2	U09759
JNK3	AA992006
ERK1	M76585
BMK α, β, γ	U29725-U29727

Agent (Gene product)	GenBank accession no.
3. Gonad differentiation	
DAX1	U31929
SOX9	Z46629
WT1	X51630
SRY	L10101
Ad4BP/SF-1	D84206-D84209
EMX2	X68880
4. Oncogenes	
c-Fos	K00650/M16287
c-Myc	J00120/K01908
Bcl-2	M13994-M13995
Bax α, β, γ	L22473-L22475
Bax δ	U19599
Bcl-x	U72398
5. Receptor-type kinase	
NGF receptor	M14764
FGF receptor	M34641
VEGF receptor	AF016050
PDGF receptor	M21616
CSF1 receptor	M33208-M33210
EGF receptor	M29366
insulin receptor	M10051

Fragments of about 1 kb that contain the 3'-untranslated regions of cDNAs for these genes were prepared as follows.

Briefly, cDNA fragments of interest were amplified by reverse transcription PCR (RT-PCR) using mRNAs from cells or tissues derived from humans or mice (Clontech) as templates. The amplified cDNAs were

confirmed to be the fragments of interest by analyzing their base sequences. The amplified fragments were recovered by ethanol precipitation and dissolved in 10 mM carbonate buffer (pH 9.5) at a concentration of 1 μM. In addition, β-actin gene as a housekeeping gene and a plasmid pUC18 as a negative control were similarly prepared. These fragments were spotted onto a slide glass with introduced amino groups (Sigma) using an instrument for preparing DNA chips (GMS) and fixed by UV irradiation. The slide glass was washed with 0.2% SDS followed by distilled water and dried to prepare a DNA array.

Example 2

(1) Administration into mouse

10 female mice (2 days old) were divided into a group with endocrine disruptor administration and a group without administration. Diethylstilbestrol (DES), which potentially causes endocrine disruption, was intravenously injected into each mouse in the group with administration at 0.1 mg/mouse/day for 2 days. Ovaries were removed on day 4, and mRNAs were prepared using an mRNA extraction kit (Qiagen).

cDNAs synthesis reactions were carried out using mixtures each containing about 3 μg of the mRNA, oligo-dT primer, Cy3-dUTP (Amersham) for the group with administration or Cy5-dUTP (Amersham) for the group without

administration, dNTPs and reverse transcriptase (Gibco-BRL). The mixtures were subjected to gel filtration, concentrated under reduced pressure and dissolved in 4 x SSC/0.2% SDS to prepare fluorescence-labeled cDNAs.

(2) Treatment of cultured cells

Human breast cancer MCF-7 cells were grown in DME medium containing 5% fetal bovine serum (FBS). After trypsinization, 2 x 10^5 cells were placed in each well of a 12-well culture plate. The cells were incubated in the same medium for 24 hours. After the medium was removed, the cells were cultured for 72 hours in DME medium containing 5% human serum from which steroid hormones had been removed by treatment with activated carbon-dextran in the presence or absence of 17-β estradiol at a concentration of 10 pM. The cells were recovered, and mRNAs were extracted as described in Example 2-(1).

cDNAs synthesis reactions were carried out using mixtures each containing about 3 μg of the mRNA, oligo-dT primer, Cy3-dUTP for the cells exposed to 17-β estradiol or Cy5-dUTP for the cells not exposed to 17-β estradiol, dNTPs and reverse transcriptase (Gibco-BRL). The mixtures were subjected to gel filtration, concentrated under reduced pressure and dissolved in 4 x SSC/0.2% SDS to prepare fluorescence-labeled cDNAs.

(3) Hybridization of labeled cDNA with DNA array

Equal volumes of the Cy3-labeled cDNA and the Cy5-labeled cDNA as prepared in (1) above were mixed together and heat-denatured. 5 µl of the mixture was dropped onto the DNA array as prepared in Example 1. A cover glass was placed on the mixture and the sides of the cover glass were sealed with a film. After incubation at 40-45°C for 10 hours, the cover glass was removed. The DNA array was washed in 0.2 x SSC/0.1% SDS for 30 minutes and in 0.2 x SSC for 30 minutes, and then air-dried. The fluorescent signals from the respective spots on the DNA array were analyzed using a microarray scanner (GMS). Furthermore, similar procedure was carried out for the labeled cDNAs obtained in (2) above.

As a result, significant changes in signals were observed for the ovary from the mouse administered with DES and MCF-7 cells exposed to 17-β estradiol. Thus, genes that were influenced by endocrine disruptors could be detected.

Example 3

(1) Preparation of DNA array

33 genes were selected from the genes listed in Table 3 in Example 1. The selected genes are shown in Table 4. β-Actin gene and a plasmid pBR322 were used as a housekeeping gene and as a negative control, respectively.

Table 4

Gene no.	Immobilized gene (Gene product)	Primer pair (SEQ ID NO)
1.	Smad3	1, 2
2.	VEGF receptor	3, 4
3.	ACTR	5, 6
4.	N-CoR/SMRT	7, 8
5.	efp	9, 10
6.	c-Myc-1	11, 12
7.	Vitamin D receptor	13, 14
8.	cathepsin G	Commercially available
9.	c-Myc-2	15, 16
10.	Bax	17, 18
11.	JNK1	19, 20
12.	p38	21, 22
13.	TRIP 1	23, 24
14.	ARA 70	25, 26
15.	insulin receptor	27, 28
16.	NGF receptor	Commercially available
17.	PDGF receptor	29, 30
18.	CSF1 receptor-1	Commercially available
19.	CSF1 receptor-2	31, 32
20.	FGF receptor	33, 34
21.	p38 gamma	35, 36
22.	Bcl-X	37, 38
23.	c-Myc-3	39, 40
24.	pS2 protein	41, 42
25.	lactoferrin	43, 44
26.	RIP 140	45, 46
27.	TIF2	47, 48
28.	JNK2	49, 50
29.	Bax delta	51, 52
30.	BMK-1	53, 54
31.	BMK-2	55, 56
32.	Src-1	57, 58
33.	p300/CBP	59, 60
34.	β-actin (positive control)	61, 62
35.	pBR 322 (negative control)	

cDNA fragments for these genes of about 100 b to about 1 kb were prepared using the respective primer pairs as indicated in Table 4 according to the method as

described in Example 1, and spotted to prepare a DNA array. Genes for cathepsin G, NGF receptor and CSF1 receptor were amplified using primers for the respective genes contained in Human UniGene DNA set (Research Genetics).

(2) Examination of influence by endocrine disruptor

Influences by treatment with various endocrine disruptors for 2 or 24 hours on cultured cells were examined.

Treatment for 2 hours: Human breast cancer MCF-7 cells were grown in DME medium containing 10% fetal bovine serum (FBS). After trypsinization, 2 x 10^6 cells were placed in a 10-cm dish. The cells were cultured for 24 hours in DME medium containing 5% fetal bovine serum from which steroid hormones had been removed by treatment with activated carbon-dextran. After removing the medium, the cells were cultured for 2 hour in the same medium containing 10 nM 17-β estradiol (E_2), 10 nM diethylstilbestrol (DES) or 5 μM bisphenol-A (BisA). As a control, cells which were cultured in the absence of such a chemical substance were similarly prepared. The treated cells were recovered, and total RNAs were extracted as described in Example 2(1).

Treatment for 24 hours: Human breast cancer MCF-7 cells were grown in DME medium containing 10% fetal bovine

serum (FBS). After trypsinization, 2 x 10^6 cells were placed in a 10-cm culture dish. The cells were incubated for 24 hours in the same medium. After removing the medium, the cells were cultured for 24 hours in DME medium containing 5% human serum from which steroid hormones had been removed by treatment with activated carbon-dextran in the presence of 10 nM 17-β estradiol (E_2), 10 nM diethylstilbestrol (DES) or 5 μM bisphenol-A (BisA). As a control, cells which were cultured in the absence of such a chemical substance were similarly prepared. The treated cells were recovered, and total RNAs were extracted as described in Example 2(1).

(3) The total RNAs as prepared in (2) above were treated with DNase. Reaction mixtures each containing about 100 to about 300 μg of the total RNA, 10 μl of 10 x AMV buffer (Life Science) and 10 U of DNaseI (Takara Shuzo) in a volume of 12 μl were prepared, incubated at 37°C for 10 minutes, extracted twice with phenol/chloroform, and then subjected to ethanol precipitation. The concentrations of the resulting total RNAs were determined using portions thereof.

(4) Reverse transcription reaction was carried out using one of the total RNAs as prepared in (3) above. The composition of the reaction mixture was as follows.

Reaction mixture A: about 130 μg of the total RNA,

10 µg of oligo-dT primer (Takara Shuzo) and 20 µl of water treated with diethylpyrocarbonate (DEPC, Wako Pure Chemical Industries).

Reaction mixture B: 12 µl of 5 x AMV RTase buffer (Life Science), 0.5 mM each of dATP, dCTP and dGTP, 0.2 mM dTTP, 60 U of RNase inhibitor (Takara Shuzo) and 0.1 mM Cy3-labeled dUTP (Amersham Pharmacia).

The reaction mixture A was incubated at 70°C for 10 minutes and then cooled on ice. The reaction mixture B was added thereto, and the resulting mixture was incubated at 42°C for 5 minutes. About 60 U of AMV RTase (Life Science) was added thereto. DEPC-treated water was further added to make the final volume to 60 µl. The resulting RT reaction mixture was incubated at 42°C for 70 minutes. 7.5 µl of 500 mM EDTA solution and 15 µl of 1 M sodium hydroxide were added to the reaction mixture. The mixture was incubated at 60°C for 1 hour to degrade the template RNA. After cooling to room temperature, 37.5 µl of 1 M tris-hydrochloride (pH 7.5) was added to the mixture. The solution was concentrated to 20 µl using Microcon YM-30 (Millipore), 200 µl of 10 mM tris-hydrochloride containing 1 mM EDTA was added thereto, and the resulting mixture was then concentrated to 20 µl. The thus obtained Cy3-labeled cDNA solution was used for the subsequent hybridization.

(5) The Cy3-labeled cDNA as prepared in (4) above

was heat-denatured, and the whole solution was dropped onto the DNA array as prepared in (1) above. A cover glass was placed on the spotted solution and the sides of the cover glass were sealed with a film. After incubation at 40-45°C for 10 hours, the cover glass was removed. The DNA array was washed in 0.2 x SSC/0.1% SDS for 30 minutes and in 0.2 x SSC for 30 minutes, and then air-dried. The fluorescent signals from the respective spots on the DNA array were analyzed using a microarray scanner (GMS). Representative values each obtained by dividing a fluorescence signal value for a sample treated with one of the substances by a fluorescence signal value for a control sample are shown in Table 5. In Table 5, a value greater than 1.00 indicates that the gene expression is promoted by the treatment with the substance. A value smaller than 1.00 indicates that the gene expression is suppressed by the treatment with the substance. A value equal to 1.00 indicates that the gene is not influenced by the test substance.

Table 5

Immobilized gene (Gene product)	DES treatment 2hr / 24hr	BisA treatment 2hr / 24hr	E_2 treatment 2hr / 24hr
Nuclear receptor or nuclear receptor transcriptional coupling			
p300/CBP	1.28 / 4.45	1.50 / 1.07	1.67 / 3.39
N-CoR/SMRT	0.62 / 1.42	1.17 / 1.08	1.86 / 1.51
ACTR	1.14 / 4.97	0.47 / 1.03	1.19 / 3.27
RIP 140	1.74 / 2.51	1.70 / 1.14	1.46 / 2.34
TIF2	1.19 / 3.04	2.66 / 0.84	2.17 / 3.30
ARA 70	0.63 / 1.37	1.31 / 0.93	1.47 / 1.45
Kinase-type signal transduction			
JNK2	0.72 / 1.85	1.30 / 0.71	1.86 / 1.71
BMK-2	1.15 / 6.06	1.04 / 0.29	1.13 / 0.05
Oncogenes			
c-Myc-1	1.08 / 0.00	1.40 / 1.30	2.52 / 1.89
Bax	1.34 / 2.27	2.41 / 1.44	1.32 / 0.99
Receptor-type kinase			
PDGF receptor	0.65 / 3.04	1.25 / 1.19	1.63 / 2.89
VEGF receptor	1.15 / 3.27	0.37 / 0.46	2.13 / 2.94

In many cases, abnormal reproduction in wild animals and reduced spermatogenesis in humans presumably caused by disruption by endocrine disrupting activities are considered to be due to suppression or interruption of signals for endocrine action at a certain stage. Therefore, it is considered that genes of which the expression is suppressed when compared with the expression in control cells should be noticed in addition to overexpressed genes. For example, among the genes used in this example, promotion of the expression of many genes that are

considered to be closely related to the action of estrogen (e.g., genes for p300/CBP, ACTR, RIP 140, TIF2, PDGF receptor and VEGF receptor) by stimulation with E_2 was observed as shown in Table 5, although little is known about the pathway, the mechanism or the like. The treatment with DES, which causes endocrine disruption, for 24 hours activated almost all of the genes other than c-Myc. Suppression of the expression of the genes for N-CoR/SMRT and ARA 70 (nuclear receptor or nuclear receptor transcriptional coupling), p38 gamma (data not shown) and JNK2 (kinase-type signal transduction), and PDGF receptor (receptor-type kinase) was observed for the treatment with DES for 2 hours. On the other hand, the treatment with BisA, which is suspected to have an endocrine disrupting activity, for 2 hours suppressed the expression of the genes for ACTR (nuclear receptor or nuclear receptor transcriptional coupling) and VEGF receptor (receptor-type kinase). The influence by the treatment with BisA on the genes was considered to be less than that observed for the stimulation with DES. The treatment with BisA for 24 hours suppressed the expression of the genes for JNK2 and BMK2 (kinase-type signal transduction), and VEGF receptor (receptor-type kinase). Thus, control of gene expression by BisA treatment in a manner different from that observed for the stimulation with DES was observed. As described

above, the use of the chip of the present invention provides a method in which significant variation of signals for expression depending on the substances used for treatment and the length of treatment time is observed. A gene that is influenced by an endocrine disruptor, in particular, of which the expression is suppressed by an endocrine disruptor, could be clearly detected.

Industrial Applicability

As described above, the method of the present invention is excellently effective in that it can detect a number of genes that are influenced by endocrine disruptors simultaneously, in vitro, rapidly and with high sensitivity. Furthermore, the present invention provides a DNA array which can be used to detect genes that are influenced by endocrine disruptors rapidly and with high sensitivity. The method of the present invention is also useful for detecting a gene involved in a previously unknown signal transduction pathway. In addition, the present invention is excellently effective in that the presence or the absence of an endocrine disruptor or a substance that potentially causes endocrine disruption can be judged using the expression of a number of genes that are influenced by endocrine disruptors obtained according to the method of the present invention as an index.

Sequence Listing Free Text

SEQ ID NO:1: Designed oligonucleotide primer to amplify Smad3 mRNA.

SEQ ID NO:2: Designed oligonucleotide primer to amplify Smad3 mRNA.

SEQ ID NO:3: Designed oligonucleotide primer to amplify VEGF receptor mRNA.

SEQ ID NO:4: Designed oligonucleotide primer to amplify VEGF receptor mRNA.

SEQ ID NO:5: Designed oligonucleotide primer to amplify ACTR mRNA.

SEQ ID NO:6: Designed oligonucleotide primer to amplify ACTR mRNA.

SEQ ID NO:7: Designed oligonucleotide primer to amplify N-CoR/SMRT mRNA.

SEQ ID NO:8: Designed oligonucleotide primer to amplify N-CoR/SMRT mRNA.

SEQ ID NO:9: Designed oligonucleotide primer to amplify efp mRNA.

SEQ ID NO:10: Designed oligonucleotide primer to amplify efp mRNA.

SEQ ID NO:11: Designed oligonucleotide primer to amplify c-Myc-1 mRNA.

SEQ ID NO:12: Designed oligonucleotide primer to

amplify c-Myc-1 mRNA.

SEQ ID NO:13: Designed oligonucleotide primer to amplify vitamin D receptor mRNA.

SEQ ID NO:14: Designed oligonucleotide primer to amplify vitamin D receptor mRNA.

SEQ ID NO:15: Designed oligonucleotide primer to amplify c-Myc-2 mRNA.

SEQ ID NO:16: Designed oligonucleotide primer to amplify c-Myc-2 mRNA.

SEQ ID NO:17: Designed oligonucleotide primer to amplify Bax mRNA.

SEQ ID NO:18: Designed oligonucleotide primer to amplify Bax mRNA.

SEQ ID NO:19: Designed oligonucleotide primer to amplify JNK1 mRNA.

SEQ ID NO:20: Designed oligonucleotide primer to amplify JNK1 mRNA.

SEQ ID NO:21: Designed oligonucleotide primer to amplify p38 mRNA.

SEQ ID NO:22: Designed oligonucleotide primer to amplify p38 mRNA.

SEQ ID NO:23: Designed oligonucleotide primer to amplify TRIP 1 mRNA.

SEQ ID NO:24: Designed oligonucleotide primer to amplify TRIP 1 mRNA.

SEQ ID NO:25: Designed oligonucleotide primer to amplify ARA 70 mRNA.

SEQ ID NO:26: Designed oligonucleotide primer to amplify ARA 70 mRNA.

SEQ ID NO:27: Designed oligonucleotide primer to amplify insulin receptor mRNA.

SEQ ID NO:28: Designed oligonucleotide primer to amplify insulin receptor mRNA.

SEQ ID NO:29: Designed oligonucleotide primer to amplify PDGF receptor mRNA.

SEQ ID NO:30: Designed oligonucleotide primer to amplify PDGF receptor mRNA.

SEQ ID NO:31: Designed oligonucleotide primer to amplify CSF1 receptor-2 mRNA.

SEQ ID NO:32: Designed oligonucleotide primer to amplify CSF1 receptor-2 mRNA.

SEQ ID NO:33: Designed oligonucleotide primer to amplify FGF receptor mRNA.

SEQ ID NO:34: Designed oligonucleotide primer to amplify FGF receptor mRNA.

SEQ ID NO:35: Designed oligonucleotide primer to amplify p38 gamma mRNA.

SEQ ID NO:36: Designed oligonucleotide primer to amplify p38 gamma mRNA.

SEQ ID NO:37: Designed oligonucleotide primer to

amplify Bcl-X mRNA.

SEQ ID NO:38: Designed oligonucleotide primer to amplify Bcl-X mRNA.

SEQ ID NO:39: Designed oligonucleotide primer to amplify c-Myc-3 mRNA.

SEQ ID NO:40: Designed oligonucleotide primer to amplify c-Myc-3 mRNA.

SEQ ID NO:41: Designed oligonucleotide primer to amplify pS2 protein mRNA.

SEQ ID NO:42: Designed oligonucleotide primer to amplify pS2 protein mRNA.

SEQ ID NO:43: Designed oligonucleotide primer to amplify lactoferrin mRNA.

SEQ ID NO:44: Designed oligonucleotide primer to amplify lactoferrin mRNA.

SEQ ID NO:45: Designed oligonucleotide primer to amplify RIP 140 mRNA.

SEQ ID NO:46: Designed oligonucleotide primer to amplify RIP 140 mRNA.

SEQ ID NO:47: Designed oligonucleotide primer to amplify TIF2 mRNA.

SEQ ID NO:48: Designed oligonucleotide primer to amplify TIF2 mRNA.

SEQ ID NO:49: Designed oligonucleotide primer to amplify JNK2 mRNA.

SEQ ID NO:50: Designed oligonucleotide primer to amplify JNK2 mRNA.

SEQ ID NO:51: Designed oligonucleotide primer to amplify Bax delta mRNA.

SEQ ID NO:52: Designed oligonucleotide primer to amplify Bax delta mRNA.

SEQ ID NO:53: Designed oligonucleotide primer to amplify BMK-1 mRNA.

SEQ ID NO:54: Designed oligonucleotide primer to amplify BMK-1 mRNA.

SEQ ID NO:55: Designed oligonucleotide primer to amplify BMK-2 mRNA.

SEQ ID NO:56: Designed oligonucleotide primer to amplify BMK-2 mRNA.

SEQ ID NO:57: Designed oligonucleotide primer to amplify Src-1 mRNA.

SEQ ID NO:58: Designed oligonucleotide primer to amplify Src-1 mRNA.

SEQ ID NO:59: Designed oligonucleotide primer to amplify p300/CBP mRNA.

SEQ ID NO:60: Designed oligonucleotide primer to amplify p300/CBP mRNA.

SEQ ID NO:61: Designed oligonucleotide primer to amplify β-actin mRNA.

SEQ ID NO:62: Designed oligonucleotide primer to

amplify β-actin mRNA.